Virology 476 (2015) 316–322

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Antiviral effect of sulfated *Chuanmingshen violaceum* polysaccharide in chickens infected with virulent Newcastle disease virus

Xu Song [a,1], Yuetian Zhang [a,1], Zhongqiong Yin [a,*,1], Xinghong Zhao [a], Xiaoxia Liang [a], Changliang He [a], Lizi Yin [a], Cheng Lv [a], Ling Zhao [a], Gang Ye [a], Fei Shi [a], Gang Shu [a], Renyong Jia [a,b,*]

[a] Natural Medecine Research Center, College of Veterinary Medicine, Sichuan Agricultural University, Ya'an, 625014, China
[b] Key Laboratory of Animal Disease and Human Health of Sichuan Province, Sichuan Agricultural University, Ya'an, 625014, China

ARTICLE INFO

Article history:
Received 17 July 2014
Returned to author for revisions
8 December 2014
Accepted 19 December 2014
Available online 9 January 2015

Keywords:
Chuanmingshen violaceum polysaccharide
Sulfated polysaccharides
Newcastle disease virus
Antiviral activity

ABSTRACT

Newcastle disease virus (NDV) belonging to the *Paramyxovirinae* subfamily is one of the most devastating pathogens in poultry. Although vaccines are widely applied to control the infection, outbreaks of Newcastle disease (ND) repeatedly happen. Currently, there are no alternative control measures available for ND. In the present study, we found that sulfated *Chuanmingshen violaceum* polysaccharide (sCVPS) were potent inhibitors of NDV in specific pathogen free chickens infected with a virulent strain. With sCVPS treatment, the survival rate increased by almost 20% and virus titers in test organs, including brain, lung, spleen and thymus, were significantly decreased. The sCVPS also exhibited the ability to prevent viral transmission by reducing the amount of virus shed in saliva and feces. Higher concentrations of interferon α and γ in serum were detected in chickens treated with sCVPS, indicating that one of the antiviral mechanisms may be attributed to the property of immunoenhancement. Histopathological examination showed that sCVPS could alleviate the tissue lesions caused by NDV infection. These results suggest that sCVPS are expected to be a new alternative control measure for NDV infection and further studies could be carried out to evaluate the antiviral activity of sCVPS against other paramyxoviruses.

Introduction

Paramyxoviruses within the *Paramyxoviridae* family are enveloped, negative-sense single-stranded RNA viruses, which comprise many important human and animal pathogens, including Measles virus, Mumps virus, Newcastle disease virus (NDV), Parainfluenza viruses, respiratory Syncytial virus, and Hendra and Nipah viruses (Aguilar and Lee, 2011; Bhella et al., 2002; Bousse et al., 2004). These pathogens cause many well-known diseases, including rubeola, parotitis and some of the deadliest zoonoses, leading to a significant number of deaths every year (Aguilar and Lee, 2011). Newcastle disease (ND), caused by infections of virulent NDV, is regarded as the fourth-most important animal disease in terms of the lost number of livestock and can cause enormous economic loss due to its highly contagious, worldwide distribution and high flock mortality (Kapczynski et al., 2013; Miller et al., 2010). There is no antiviral drug available for ND and so far the only recommended prevention method is vaccination (Elizondo-Gonzalez et al., 2012). However, there are significant differences between the current vaccine strains and prevailing NDV strains, which induce continual outbreaks of ND even in vaccinated poultry flocks (Zhang et al., 2011). Thus, it is urgent to find new alternative control measures.

Sulfated polysaccharides have sulfate groups esterified with the polysaccharide hydroxyl groups and exhibit multiple bioactivities such as antiviral, anticancer, antioxidative, anticoagulant and immune-strengthening effects (Jiménez-Escrig et al., 2011; Jiao et al., 2011; Nguyen et al., 2012; Wijesekara et al., 2011; Moura Neto et al., 2014). It has been over 50 years since the first report on the antiviral activity of sulfated polysaccharides (Gerber et al., 1958). Varieties of sulfated polysaccharides that possess a wide spectrum of antiviral properties have been separated from different plants or synthesized (Pujol et al., 2007; Witvrouw and De Clercq, 1997; Chrestani et al., 2009; Chen et al., 2014; Ghosh et al., 2009). Moreover, some of them exhibit the potential ability to inhibit the infection of Paramyxoviruses (Song et al., 2013c). The mechanism of action can be ascribed to the interaction of sulfated polysaccharides with viral envelope glycoproteins leading to prevention of virus entry (Damonte et al., 2004; Harden et al., 2009; Pujol et al., 2007).

* Corresponding authors. Fax: +86 835 2885302.
E-mail addresses: yinzhongq@163.com (Z. Yin), cqrc_jry@163.com (R. Jia).
[1] These authors contribute equally to this work and should be considered as the first author.

http://dx.doi.org/10.1016/j.virol.2014.12.030
0042-6822/

As it has been demonstrated that some sulfated polysaccharides exhibit potent antiviral activity, it would be expected that there are more candidates with antiviral properties by sulfated modification of polysaccharides. Therefore, we previously have studied polysaccharides extracted from *Chuanmingshen violaceum* (CVPS), and found that sulfated *Chuanmingshen violaceum* polysaccharides (sCVPS) possessed remarkable inhibitory effect of duck enteritis virus (Song et al., 2013b) and NDV (Song et al., 2013c), while non-sulfated polysaccharides did not show any activity. Since the promising antiviral activity of sCVPS that was detected in vitro, this study was conducted to evaluate whether sCVPS could show antiviral activity in specific pathogen free (SPF) chickens infected with a virulent NDV strain in order to develop a new alternative control measure for NDV infection.

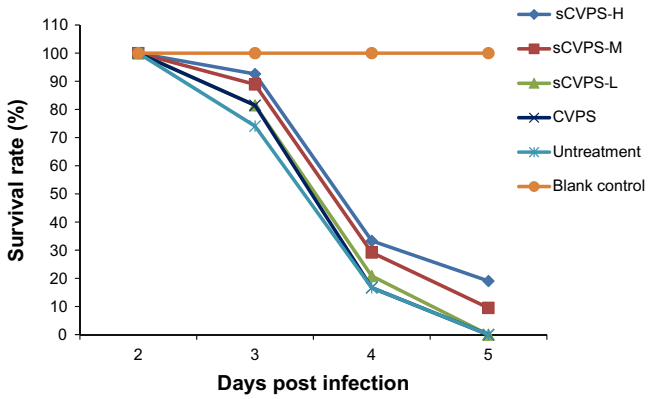

Fig. 1. The survival rate of chickens in each group. The number of alive chickens in each group was recorded at 2, 3, 4 and 5 dpi, respectively. The survival rate of each group was then calculated according to the formula: survival rate=the number of alive chickens/the total number of chickens. At 5 dpi, the significant difference ($P<0.05$) was observed between the sCVPS-H group and the untreated group according to the results of the Chi-square test.

Results

Survival rate

The survival rate of chickens in each group is shown in Fig. 1. There were no deaths in each group at 2 days post-infection (dpi), but after 3 dpi the chickens began to die. The survival rates of chickens treated with sCVPS were higher than those in the untreated group and the CVPS-treated group at 3 dpi and 4 dpi. The high dose of sCVPS-treated (sCVPS-H) group exhibited the highest survival rate, decreasing from 92.59% at 3 dpi to 33.33% at 4 dpi, while the survival rate of the untreated group fell from 74.07% at 3 dpi to 16.67% at 4 dpi. In the CVPS-treated group, the survival rate was a little higher than that in the untreated group at 3 dpi, but it declined to the same value (16.67%) at 4 dpi. In addition, the chickens in the medium dose of sCVPS-treated (sCVPS-M) group had a higher survival rate than low dose of sCVPS-treated (sCVPS-L) group. At 5 dpi, no chickens were alive except in sCVPS-H group and sCVPS-M group. The survival rates were approximately 20% and 10%, respectively.

Virus titers

To test whether sCVPS and CVPS could reduce virus amount in chickens, several tissues, including lung, spleen, thymus and brain were collected and the virus titers of these tissues were determined by measuring the $TCID_{50}$ values (Fig. 2). The virus replicated more rapidly in lung, spleen and thymus when compared with the brain

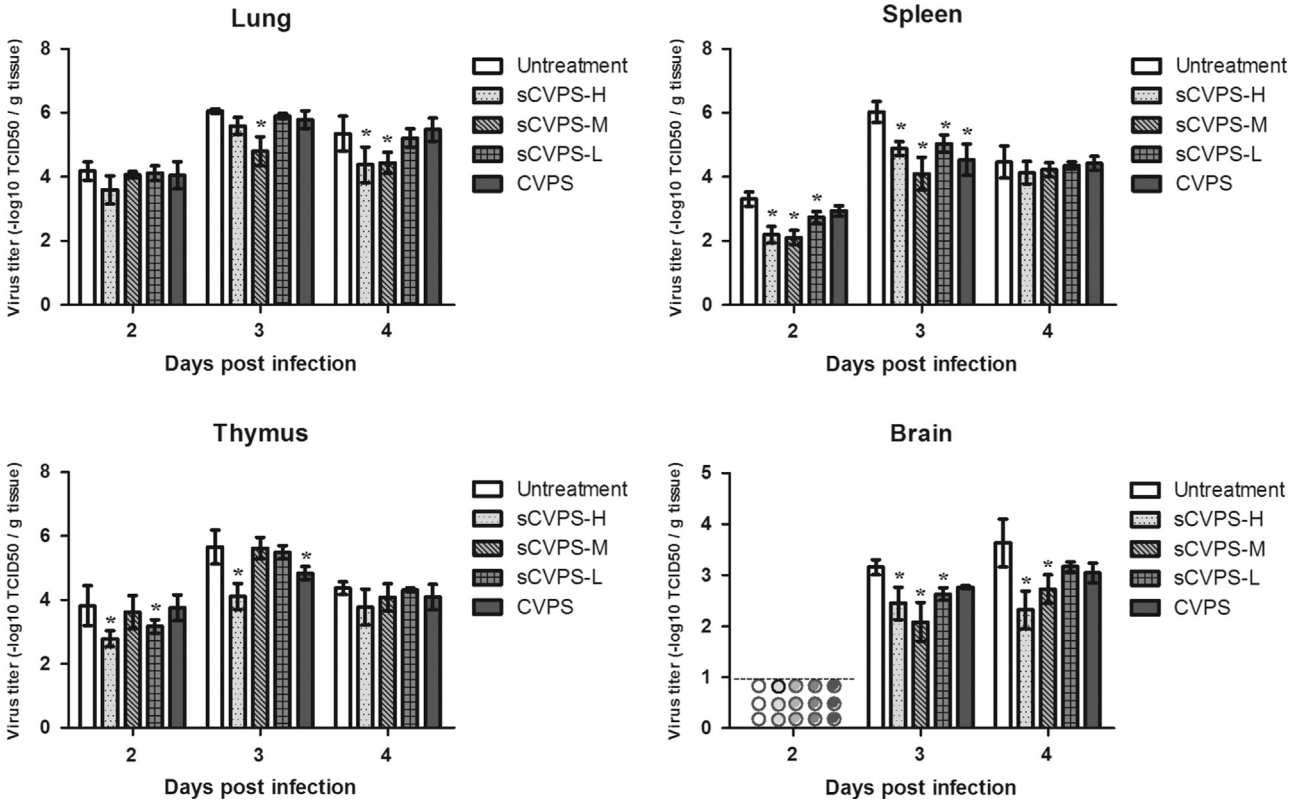

Fig. 2. Virus titers of test organs in each group. The lung, spleen, thymus and brain were collected at 2, 3 and 4 dpi. After homogenate, the supernatant was used to determine the virus titer on chicken embryo fibroblasts. The dotted line in the brain at 2 dpi showed the detection limit. The virus titers of these brain samples which was lower than detection limit was inoculated into embryonated eggs for virus positive test. A cycle symbol indicated a brain sample was virus positive. "*" represents the significant differences ($P<0.05$) observed between the untreated group and the treated groups.

tissue. Moreover, in these three organs, the virus titers reached the maximum values at 3 dpi and then declined. The virus titer in the brain continually increased over the experimental period. After sCVPS-treatment, the virus titers were reduced significantly in the test organs; the CVPS control treatment did not show the same potent effects on inhibiting virus proliferation. The virus titers from chickens treated with the high and medium doses of sCVPS were lower than those in sCVPS-L group. The spleen and thymus of the sCVPS-treatment group exhibited a significant decrease in the virus titers ($P<0.05$) at 2 and 3 dpi, but no significant differences were observed at 4 dpi. However, the virus titers of lung and brain did not decline significantly at 2 dpi, but significant decreases ($P<0.05$) were observed at 3 and 4 dpi.

Virus shedding

The virus-shedding assay was designed to evaluate the role of sCVPS and CVPS in reducing the transmissibility in NDV-infected chickens. Therefore, oropharyngeal and cloacal swabs were collected from sCVPS and CVPS treated and untreated chickens at 2, 3 and 4 dpi, the virus titers were then determined by measuring the $TCID_{50}$ values (Fig. 3). The amount of virus shed in oropharyngeal and cloacal swabs was reduced when treated with sCVPS. After treatment with sCVPS-H and sCVPS-M, the amount of virus shed in oropharyngeal swabs at 3 dpi as well as in cloacal swabs at 4 dpi were significantly reduced ($P<0.05$). Moreover, in the sCVPS-H-treated group, the amount of virus from oropharyngeal swabs at 4 dpi as well as in cloacal swabs at 3 dpi was significantly decreased ($P<0.05$). However, CVPS and sCVPS-L exhibited little effect in inhibiting virus shedding.

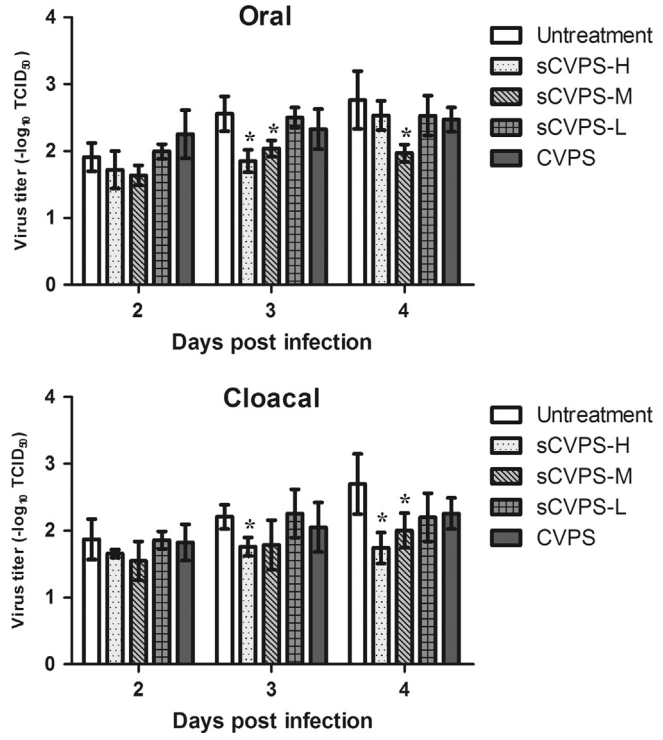

Fig. 3. Virus titers of oral and cloacal swabs collected from each group on 2, 3 and 4 dpi. The virus titers were determined on chicken embryo fibroblasts. "*" represents the significant differences ($P<0.05$) observed between the untreated group and the treated groups.

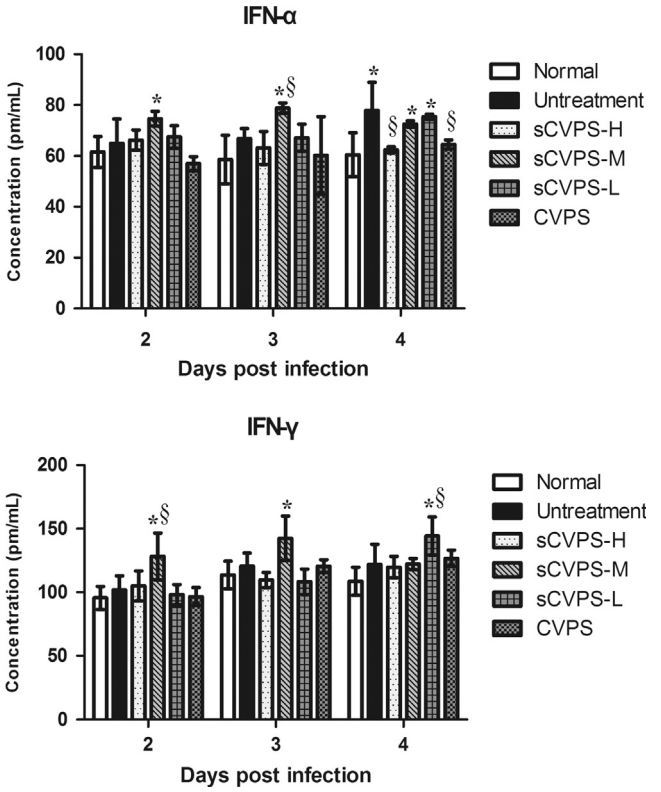

Fig. 4. The concentrations of interferon-α and γ in serum obtained from each group at 2, 3 and 4 dpi. The interferon concentrations were determined by ELISA. "*" represents the significant differences ($P<0.05$) observed between the normal group and the NDV-infected groups. "§"represents the significant differences ($P<0.05$) observed between the untreated group and the treated groups.

The changes of serum cytokines

It is widely reported that polysaccharides possess the ability to enhance the body's immunity, thus the concentrations of serum cytokine possessing antiviral property, including IFN-α and IFN-γ were determined by ELISA. As shown in Fig. 4, the concentrations of IFN-α and IFN-γ were increased after NDV infection when compared with uninfected group. The concentrations of IFN-α and IFN-γ of NDV-infected chickens treated with sCVPS were higher than those in untreated chickens. Chickens treated with a medium dose of sCVPS exhibited significantly improved production of IFN-α (throughout the experiment) and IFN-γ (until 4 dpi) when compared with the uninfected group ($P<0.05$). The production of IFN-α and IFN-γ in sCVPS-L group was also significantly augmented when compared with uninfected group ($P<0.05$). In addition, the IFN-α concentration of chickens treated with the medium dose of sCVPS was significantly higher than that in the untreated group at 3 dpi ($P<0.05$); the IFN-γ concentrations in sCVPS-M group at 2 dpi as well as sCVPS-L group at 4 dpi were also higher than in the untreated group ($P<0.05$).

Histopathology study

NDV infections always cause severe lesions in various organs. Consequently, a histopathology assay was conducted to assess the pathological changes and whether treatment with sCVPS could alleviate the lesions. As shown in Fig. 5, virus infection induced serious necrosis in the spleen (Fig. 5B); with sCVPS treatment, the main histopathological changes were hyperemia and focal necrosis (Fig. 5C). In the thymus, nearly complete necrosis of the cortex was observed in the untreated group (Fig. 5E); little necrosis and congestion were detected in sCVPS-treated group (Fig. 5F). In the

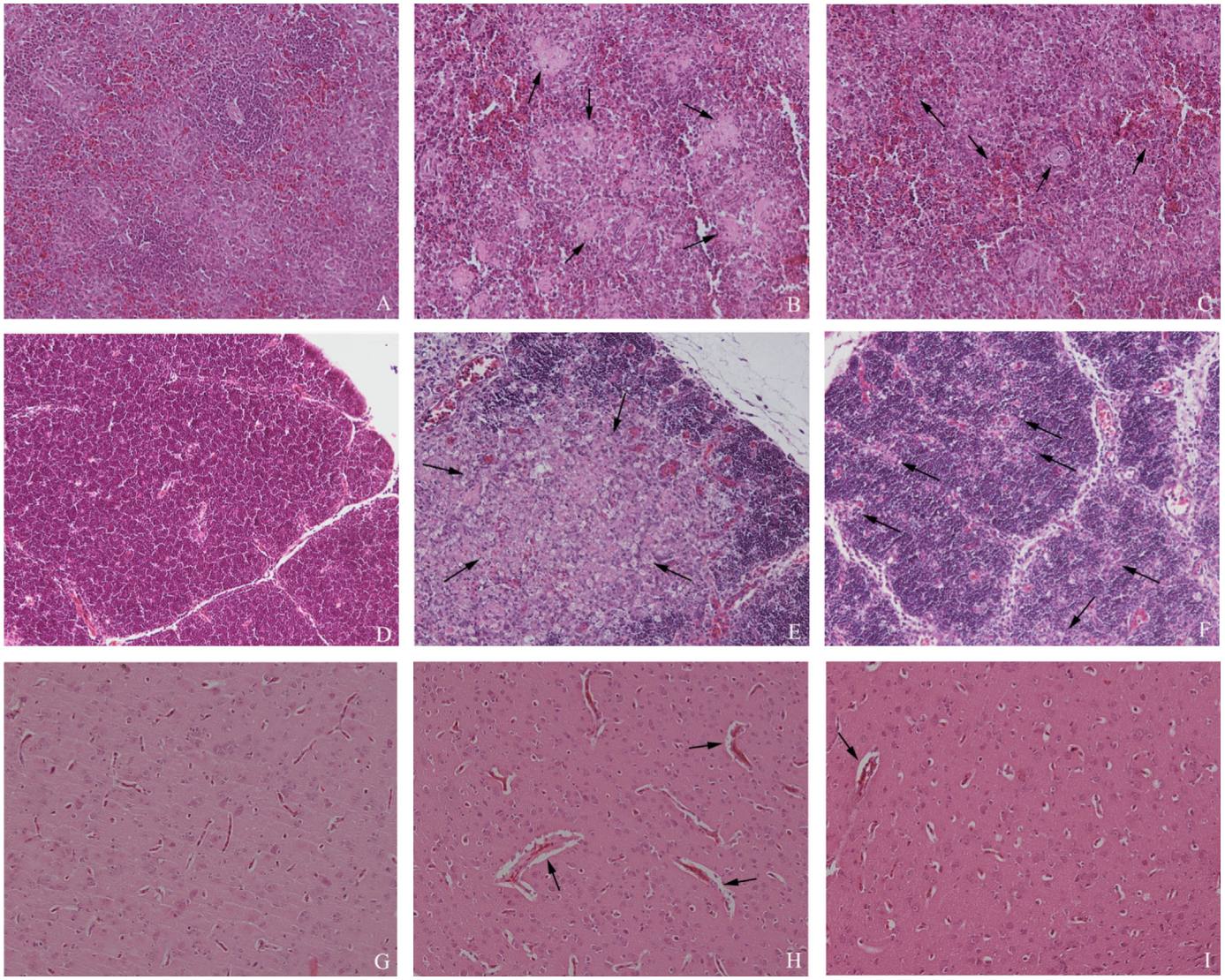

Fig. 5. The histopathological changes of the CVPS-H, untreated and normal groups. In spleen (A–C, ×200), NDV infection induced serious necrosis (B, denoted by arrowhead); with sCVPS treatment, the main histopathological changes were hyperemia and focal necrosis (C, denoted by arrowhead); A, normal group. In thymus (D–F, ×200), serious necrosis induced by NDV was observed (E, denoted by arrowhead); little necrosis and congestion were detected in sCVPS-treated group (F, denoted by arrowhead); D, normal group. In brain (G–I, ×200), the lesions were illustrated by hyperemia (H, denoted by arrowhead); sCVPS could alleviate the lesion (I, denoted by arrowhead); G, normal group.

Table 1
Lesional scores for each group on 4 dpi*

Group	Spleen	Thymus	Brain	Liver	Lung	Heart	Kidney
CVPS-H	3.56 ± 0.51^{c}	5.44 ± 0.51^{b}	0.44 ± 0.20^{b}	1.33 ± 0.34^{a}	3.22 ± 0.39^{c}	2.00 ± 0.58^{a}	2.22 ± 0.19^{c}
CVPS-M	4.45 ± 0.39^{c}	7.33 ± 0.58^{a}	0.56 ± 0.20^{b}	1.22 ± 0.39^{a}	4.00 ± 0.67^{bc}	1.78 ± 0.51^{a}	3.11 ± 0.70^{bc}
CVPS-L	6.00 ± 1.00^{b}	7.67 ± 1.15^{a}	1.11 ± 0.19^{a}	1.56 ± 0.20^{a}	4.78 ± 0.51^{ab}	1.44 ± 0.20^{a}	4.56 ± 0.84^{a}
CVPS	6.67 ± 0.58^{a}	8.00 ± 1.00^{a}	1.00 ± 0.33^{a}	2.00 ± 0.33^{a}	5.11 ± 0.77^{ab}	1.56 ± 0.53^{a}	4.22 ± 0.77^{ab}
Untreatment	7.67 ± 0.58^{a}	8.67 ± 0.58^{a}	1.44 ± 0.19^{a}	1.89 ± 0.51^{a}	5.00 ± 0.33^{a}	1.67 ± 0.34^{a}	4.89 ± 0.38^{a}

[a,b,c]Significant differences exist when there are not the same letters on each column ($P < 0.05$).

* Lesional scores of each organ were obtained by multiplying the degree of severity (0 = no lesions, 1 = mild lesions, 2 = moderate lesions, and 3 = severe lesions) with the extent of lesions (1 = low extent, 2 = intermediate extent, and 3 = large extent).

brain, capillary hyperemia was found in the untreated group (Fig. 5H); with sCVPS treatment, the lesions became milder (Fig. 5I). These observations were also proved by the lesional score (Table 1). When compared with the untreated group, the significantly lower lesional scores on spleen, thymus, brain, lung and kidney were observed in the sCVPS-treated groups except CVPS-L group ($P < 0.05$). No significant differences were observed in lesional scores on liver and heart between the sCVPS-treated and untreated groups. CVPS did not show any activity to inhibit the histologic lesions caused by NDV infection.

Discussion

It has been over 50 years since live and inactivated vaccines made by lentogenic strains, including LaSota, B1 and F were used

for NDV control (Han et al., 2008; Miller et al., 2009a). These vaccine strains belonging to genotypes (I and II) are divergent from the current circulating strains (mainly genotypes VI and VII) in their biology, serology and genetics, so poultry cannot achieve the desirable immunity, leading to outbreaks occurring year after year (Dortmans et al., 2012; Alexander, 2001; Rui et al., 2010). The chemotherapeutic drug ribavirin showed potent inhibitory effects against many paramyxoviruses, but it exhibited very poor antiviral activity against NDV (Elizondo-Gonzalez et al., 2012). In the past few years, there has been much research focused on developing an alternative control measure from sulfated polysaccharides, but results only showed in vitro activity (Wang et al., 2010; Zhao et al., 2010; Nguyen et al., 2012; Elizondo-Gonzalez et al., 2012). In the present study, we first evaluated the anti-NDV potency of sulfated polysaccharides in vivo and found that sCVPS was capable of protecting chickens from NDV infection and improving the survival rate by almost 20% in a dose-dependent manner, but non-sulfated CVPS exhibited no anti-NDV activity (Fig. 1). These results suggested higher amounts of sCVPS for treatment. Infection with virulent NDV always causes rapid death in susceptible poultry, nearly 100% mortality within 3–6 dpi (Wakamatsu et al., 2006). In our study we observed the same result, with no chickens surviving at 5 dpi in the untreated group (Fig. 1). This study also provided in vivo evidence for the theory that sulfated modification could improve the antiviral activity of polysaccharides.

NDV starts its replication at the site of infection and is then transported to other organs through blood, except for lentogenic strains (Liu et al., 2012; Kapczynski et al., 2013). Virus titers are expected to be an indicator of viral proliferation and an index for assessing the inhibitory effect of sCVPS. This study found that sCVPS-treatment could reduce the viral load in the test organs (Fig. 2), and virus titers in the spleen and thymus were significantly decreased earlier than those in the lung and brain. The results suggest that the immune organs may act as the target site for sCVPS in vivo. In addition, our results are consistent with the observations that the viral reproduction reached peak at 3 dpi in organs except for the brain (Dortmans et al., 2011).

In NDV-infected birds, large amounts of viruses are excreted into the environment through secretions including fine aerosols, large droplets and feces. The spread of NDV may then take place by inhalation or ingestion of these secretions (Saif et al., 2008). Thus, there is an opportunity to minimize the impact of an outbreak and inhibit the transmission of disease if a reduction in virus shedding is achieved (Miller et al., 2009b). However, recent studies have revealed that classic vaccines showed lower ability to reduce viral shedding than the vaccines formulated with the homologous genotype of currently circulating viruses which is not commercially available (Miller et al., 2007; 2009b; 2013). Our results have demonstrated that sCVPS could significantly decrease the amount of virus shed in saliva and feces (Fig. 3). This property may enable sCVPS to be used for the control of NDV transmission. In addition, we also found that infection with virulent NDV could induce viral shedding that starts at 2 dpi, which consists with previous findings (Miller et al., 2007, 2009b, 2013). There were other studies showing that the onset of viral shedding was at 3 dpi in chickens and pigeons, indicating that these differences may be due to varying replication efficiencies (Guo et al., 2014).

The immune system plays a key role in protecting the body against foreign pathogens through many ways including innate immunity and acquired immunity. The cell-mediated immunity induced by infection of virulent NDV is probably negligible due to the rapid death of birds, so the innate immune response is the primary contributor to the inhibition of virus growth and spread (Kapczynski et al., 2013; Miller et al., 2013). Type I and II interferon (IFN α and γ) are in the front line in inhibiting virus infections, thus up-regulation of these cytokines is critical for antiviral immunity (Mibayashi et al., 2007; Shrestha et al., 2006). In this study, treatment with the medium dose of sCVPS resulted in a significant increase of IFN α and γ concentration, but the high dose group appeared to be the same level with the untreated group (Fig. 4). These results suggest that the medium dose of sCVPS exhibited its inhibitory effect by immunoenhancement while the antiviral activity of high dose of sCVPS was achieved by another pathway such as directly inactivating viruses.

According to the pathogenicity, NDV strains are usually divided into three pathotypes: lentogenic, mesogenic and velogenic strains (Snoeck et al., 2009). The velogenic viruses are further classified into vicerotropic and neurotropic strains based on different clinical signs (Snoeck et al., 2009). The main histopathological characteristic related to infection of vicerotropic strain is serious necrosis in lymphoid tissues, especially in the spleen and gut-associated lymphoid tissue (Cattoli et al., 2011). In contrast, the neurotropic strain predominately induces lesions of central nervous system, which consist of hypertrophy/hyperplasia of vascular endothelium, moderate gliosis, and multifocal necrosis of the Purkinje cells (Cattoli et al., 2011). Thus spleen, thymus and brain were selected as the target organs to evaluate the tissue lesions. This study also scored histological lesions for each organ, which allowed us to evaluate the changes of the lesions by quantitative data. Our results showed that mild lesions were observed in the chickens treated with the high dose of sCVPS, which suggested that sCVPS could slow down the histopathological changes (Fig. 5 and Table 1). In addition, severe necrosis in spleen (Fig. 5B) and thymus (Fig. 5E) was observed; only mild cerebral lesions were observed (Fig. 5H). These results suggested that F48E9 strain belongs to vicerotropic pathotype.

In conclusion, sCVPS possess the ability to inhibit NDV infection in chickens by promoting survival rate, reducing virus titer and viral shedding, enhancing immune response and alleviating tissue lesions. With the promising in vivo anti-NDV properties reported in this study, sCVPS exhibits potential for NDV control, and further studies should be conducted for evaluating the antiviral activity against other paramyxoviruses.

Materials and methods

Preparation of sCVPS and CVPS

CVPS was separated by decoction as previously described (Song et al., 2013a). The content of polysaccharides was 93.85%. It was sulfated by the chlorosulfonic acid-pyridine method to yield sCVPS with 0.95 degree of sulfation (Song et al., 2013b). CVPS and sCVPS were dissolved in sterile physiological saline, respectively, and then the solutions were sterilized by the circulating steam method.

Virus

Newcastle disease virus (F48E9 strain) was provided by the Department of Prevention Veterinary Medicine, Sichuan Agricultural University (Ya'an, China). Viruses were propagated in 10-day-old SPF chicken embryo eggs and the 50% egg infectious dose (EID_{50}) was measured as $10^{-8.17}$/mL.

SPF chickens and experimental design

SPF fertile eggs were bought from the Beijing Laboratory Animal Centre, Beijing, China and hatched by an automatic incubator. All chickens were reared in separate biosafety level 2 isolators under uniform conditions with sufficient feed and water. The experimental protocol was approved by the National Institute of Animal Health Animal Care and Use Committee at Sichuan Agricultural University (approval number 2010-010)

At 14 days old, 180 chickens were randomly divided into six groups and challenged with 0.1 mL NDV suspension at a dose of 2×10^4 EID_{50} through nasal drip and eye-drop, except the blank control group. Then, chickens in sCVPS-H, sCVPS-M and sCVPS-L groups were administered with 0.1 mL sCVPS at concentrations of 8, 4 and 2 mg/kg · body weight, respectively; in CVPS-treated groups with 0.1 mL of CVPS (4 mg/kg · body weight); in the untreated group and blank control group with an equal volume of physiological saline. The sCVPS, CVPS and physiological saline were given through intramuscular injection, once a day for 4 successive days.

Tissue virus titers assay

Three chickens from each group were sacrificed at 2, 3 and 4 dpi, and brain, lung, spleen and thymus were collected. These organs were homogenized in Phosphate Buffer Solution (PBS), followed by centrifugation at 10,000 *g* for 20 min. The virus titers were then determined on chicken embryo fibroblasts as follows. Serial 10-fold dilutions of supernatant of tissue homogenates were prepared with cell culture media and subsequently added into cells in 96-well plates. After incubation for 2 h at 37 °C, the inoculums were aspirated and cells were washed three times with PBS. Then, culture media were added and cells were re-incubated for 72 h at 37 °C. Finally, cytopathic effects of cells in each well were observed and virus titers were calculated by the Reed–Muench method (Reed and Muench, 1938). The mean virus titers were presented as ($-\log_{10}TCID_{50}$/g tissue) (Dortmans et al., 2011).

Virus shedding assay

The virus shedding assay was performed as previously described (Miller et al., 2007). Oral and cloacal swabs were collected from three chickens per group at 2, 3 and 4 dpi and put into tubes containing 1 mL PBS supplemented with penicillin (2000 units/mL), gentamicin (200 μg/mL) and amphotericin B (4 μg/mL). After twice freezing and thawing and fully shaking, the tubes were centrifuged at 10,000 *g* for 10 min. Then, the virus titers of the supernatant were measured as in the method reported above.

Serum cytokines assay

Blood samples (three chickens per group) from jugular vein were put into sterile tubes followed by clotting at 37 °C for 30 min. Then, the serum was separated by centrifugation. The concentrations of cytokines including IFN-α and IFN-γ in serum were determined by using the ELISA kit according to the manufacturer's instructions (Bio-Swamp Immunoassay R&D Center, Shanghai). Briefly, serial 2-fold dilutions of standard cytokine were prepared with standard diluent, and then 50 μL of each dilution was added into the antibody-coated microtiter plate. The 40 μL of each serum sample together with 10 μL biotinylated antibody were added. The plate sealed by a closure membrane was allowed to incubate at 37 °C for 30 min, followed by washing five times with wash buffer. HRP-conjugate reagent was then added into each well and the plate was incubated at 37 °C for 30 min. After washing, 50 μL chromogen solution A and B were added and the plate was incubated at 37 °C for 15 min. Finally, 50 μL stop solution was added and the optical density at 450 nm was measured by a microplate reader (Bio-Rad, USA). The sample concentration was calculated according to the constructed standard curve.

Histopathological assay

The chickens from the treated and untreated groups were sacrificed at 4 dpi. A small piece of the tissues, including brain, spleen, thymus, heart, liver, lung and kidney were excised and fixed in PBS containing 10% formalin. The samples were then washed with water overnight followed by dehydration with a graded ethanol solution series and xylene. After embedding in paraffin, a 5 μm section of each tissue was procured and stained with hematoxylin – eosin for histopathological evaluation under an optical microscope. Three slides from different part of each tissue (3 chickens per group) were analyzed. The whole lesions for each tissue were scored by multiplying the degree of severity (0=no lesions, 1=mild lesions, 2=moderate lesions, and 3=severe lesions) with the extent of lesions (1=low extent, 2=intermediate extent, and 3=large extent) (Gerez et al., 2014). For each organ, the maximal lesional score was 9 and the minimal score was 0.

Acknowledgments

We thank Dr. David Grandy for checking the grammar and spelling. This study was supported by the Foundation for the Author of Excellent Doctoral Dissertation of Sichuan Agricultural University (YB2014003), the International cooperation projects of Sichuan Province (2014HH0058, 2013HH0042), the Sichuan Youth Science and Technology Innovation Research Team for waterfowl disease prevention and control (2013TD0015) and the National Natural Science Foundation of China (Grant no. 31372477).

References

Aguilar, H.C., Lee, B., 2011. Emerging paramyxoviruses: molecular mechanisms and antiviral strategies. Expert Rev. Mol. Med. 13, e6.
Alexander, D.J., 2001. Newcastle disease. Br. Poult. Sci. 42, 5–22.
Bhella, D., Ralph, A., Murphy, L.B., Yeo, R.P., 2002. Significant differences in nucleocapsid morphology within the Paramyxoviridae. J. Gen. Virol. 8, 1831–1839.
Bousse, T.L., Taylor, G., Krishnamurthy, S., Portner, A., Samal, S.K., Takimoto, T., 2004. Biological significance of the second receptor binding site of Newcastle disease virus hemagglutinin-neuraminidase protein. J. Virol. 78, 13351–13355.
Cattoli, G., Susta, L., Terregino, C., Brown, C., 2011. Newcastle disease: a review of field recognition and current methods of laboratory detection. J. Vet. Diagn. Investig. 23, 637–656.
Chen, Y., Xiong, W., Zeng, L., Wang, D., Liu, J., Wu, Y., Hu, Y., 2014. Comparison of Bush Sophora Root polysaccharide and its sulfate's anti-duck hepatitis A virus activity and mechanism. Carbohydr. Polym. 102, 333–340.
Chrestani, F., Sierakowski, M.R., de Andrade Uchoa, D.E., Nozawa, C., Sassaki, G.L., Gorin, P.A., Ono, L., 2009. In vitro antiherpetic and antirotaviral activities of a sulfate prepared from *Mimosa scabrella* galactomannan. Int. J. Biol. Macromol. 45, 453–457.
Damonte, E.B., Matulewicz, M.C., Cerezo, A.S., 2004. Sulfated seaweed polysaccharides as antiviral agents. Curr. Med. Chem. 11, 2399–2419.
Dortmans, J.C., Peeters, B.P., Koch, G., 2012. Newcastle disease virus outbreaks: vaccine mismatch or inadequate application? Vet. Microbiol. 160, 17–22.
Dortmans, J.C., Rottier, P.J.M., Koch, G., Peeters, B.P., 2011. Passaging of a Newcastle disease virus pigeon variant in chickens results in selection of viruses with mutations in the polymerase complex enhancing virus replication and virulence. J. Gen. Virol. 92, 336–345.
Elizondo-Gonzalez, R., Cruz-Suarez, L.E., Ricque-Marie, D., Mendoza-Gamboa, E., Rodriguez-Padilla, C., Trejo-Avila, L.M., 2012. In vitro characterization of the antiviral activity of fucoidan from *Cladosiphon okamuranus* against Newcastle Disease Virus. Virol. J. 9, 307.
Gerber, P., Dugene, J.D., Adams, E.V., Sherman, J.H., 1958. Protective effect of seaweed extracts for chicken embryos infected with influenza B or mumps virus. Proc. Soc. Exp. Biol. Med. 99, 590–593.
Gerez, J.R., Pinton, P., Callu, P., Grosjean, F., Oswald, I.P., Bracarense, A.P.F., 2014. Deoxynivalenol alone or in combination with nivalenol and zearalenone induce systemic histological changes in pigs. Exp. Toxicol. Pathol..
Ghosh, T., Chattopadhyay, K., Marschall, M., Karmakar, P., Mandal, P., Ray, B., 2009. Focus on antivirally active sulfated polysaccharides: from structure–activity analysis to clinical evaluation. Glycobiology 19, 2–15.
Guo, H., Liu, X., Xu, Y., Han, Z., Shao, Y., Kong, X., Liu, S., 2014. A comparative study of pigeons and chickens experimentally infected with PPMV-1 to determine antigenic relationships between PPMV-1 and NDV strains. Vet. Microbiol. 168, 88–97.
Han, G.Z., He, C.Q., Ding, N.Z., Ma, L.Y., 2008. Identification of a natural multi-recombinant of Newcastle disease virus. Virology 371, 54–60.
Harden, A.E., Falshaw, R., Carnachan, M.S., Kern, R.E., Prichard, N.M., 2009. Virucidal activity of polysaccharide extracts from four algal species against herpes simplex virus. Antivir. Res. 83, 282–289.

Jiao, G., Yu, G., Zhang, J., Ewart, H.S., 2011. Chemical structures and bioactivities of sulfated polysaccharides from marine algae. Mar. Drugs 9, 196–223.
Jiménez-Escrig, A., Gómez-Ordóñez, E., Rupérez, P., 2011. Seaweed as a source of novel nutraceuticals: sulfated polysaccharides and peptides. Adv. Food. Nutr. Res. 64, 325–337.
Kapczynski, D.R., Afonso, C.L., Miller, P.J., 2013. Immune responses of poultry to Newcastle disease virus. Dev. Comp. Immunol. 41, 447–453.
Liu, W.Q., Tian, M.X., Wang, Y.P., Zhao, Y., Zou, N.L., Zhao, F.F., Cao, S.J., Wen, X.T., Liu, P., Huang, Y., 2012. The different expression of immune-related cytokine genes in response to velogenic and lentogenic Newcastle disease viruses infection in chicken peripheral blood. Mol. Biol. Rep. 39, 3611–3618.
Mibayashi, M., Martínez-Sobrido, L., Loo, Y.M., Cárdenas, W.B., Gale Jr., M., García-Sastre, A., 2007. Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. J. Virol. 81, 514–524.
Miller, P.J., Afonso, C.L., El, Attrache, J., Dorsey, K.M., Courtney, S.C., Guo, Z., Kapczynski, D.R., 2013. Effects of Newcastle disease virus vaccine antibodies on the shedding and transmission of challenge viruses. Dev. Comp. Immunol. 41, 505–513.
Miller, P.J., Decanini, E.L., Afonso, C.L., 2010. Newcastle disease: Evolution of genotypes and the related diagnostic challenges. Infect. Genet. Evol. 10, 26–35.
Miller, P.J., Estevez, C., Yu, Q., Suarez, D.L., King, D.J., 2009b. Comparison of viral shedding following vaccination with inactivated and live Newcastle disease vaccines formulated with wild-type and recombinant viruses. Avian Dis. 53, 39–49.
Miller, P.J., Kim, L.M., Ip, H.S., 2009a. Evolutionary dynamics of Newcastle disease virus. Virology 391, 64–72.
Miller, P.J., King, D.J., Afonso, C.L., Suarez, D.L., 2007. Antigenic differences among Newcastle disease virus strains of different genotypes used in vaccine formulation affect viral shedding after a virulent challenge. Vaccine 25, 7238–7246.
Moura Neto, E., Sombra, V.G., Richter, A.R., Abreu, C.M., Maciel, J.S., Cunha, P.L., Ono, L., Sierakowski, M.R., Feitosa, J.P., de Paula, R.C., 2014. Chemically sulfated galactomannan from *Dimorphandra gardneriana* seed: characterization and toxicity evaluation. Carbohydr. Polym. 101, 1013–1017.
Nguyen, T.L., Chen, J., Hu, Y., Wang, D., Fan, Y., Wang, J., Abula, S., Zhang, J., Qin, T., Chen, X., Chen, X., Khakame, S.K., Dang, B.K., 2012. In vitro antiviral activity of sulfated *Auricularia auricula* polysaccharides. Carbohydr. Polym. 3, 1254–1258.
Pujol, A.C., Carlucci, J.M., Matulewicz, C.M., Damonte, B.E., 2007. Natural sulfated polysaccharides for the prevention and control of viral infections. Top. Heterocycl. Chem. 11, 259–281.
Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. Am. J. Hyg. 27, 493–497.
Rui, Z., Juan, P., Jingliang, S., Jixun, Z., Xiaoting, W., Shouping, Z., Xiaojiao, L., Guozhong, Z., 2010. Phylogenetic characterization of Newcastle disease virus isolated in the mainland of China during 2001–2009. Vet. Microbiol. 141, 246–257.
Saif, Y.M., Fadly, A.M., Glisson, J.R., McDougald, L.R., Nolan, L.K., Swayne, D.E., 2008. Diseases of Poultry, twelfth ed Blackwell Publishing, UK.
Snoeck, C.J., Ducatez, M.F., Owoade, A.A., Faleke, O.O., Alkali, B.R., Tahita, M.C., Tarnagda, Z., Ouedraogo, J.B., Maikano, I., Mbah, P.O., Kremer, J.R., Muller, C.P., 2009. Newcastle disease virus in West Africa: new virulent strains identified in non-commercial farms. Arch. Virol. 154, 47–54.
Shrestha, B., Wang, T., Samuel, M.A., Whitby, K., Craft, J., Fikrig, E., Diamond, M.S., 2006. Gamma interferon plays a crucial early antiviral role in protection against West Nile virus infection. J. Virol. 80, 5338–5348.
Song, X., Xu, J., Yin, Z.Q., Jia, R.Y., Cheng, A.C., Deng, Y.X., Lü, C., Liang, X.X., Wang, Y., Yang, Z.X., Yao, X.P., Zhang, W., 2013a. Effects of polysaccharide from *Chuanminshen violaceum* on immune response of Newcastle disease vaccine in chicken. Acta Sci. Vet. 41, 1104.
Song, X., Yin, Z., Li, L., Cheng, A., Jia, R., Xu, J., Wang, Y., Yao, X., Lv, C., Zhao, X., 2013b. Antiviral activity of sulfated *Chuanminshen violaceum* polysaccharide against duck enteritis virus in vitro. Antivir. Res. 98, 344–351.
Song, X., Yin, Z., Zhao, X., Cheng, A., Jia, R., Yuan, G., Xu, J., Fan, Q., Dai, S., Lu, H., Lv, C., Liang, X., He, C., Su, G., Zhao, L., Ye, G., Shi, F., 2013c. Antiviral activity of sulfated *Chuanmingshen violaceum* polysaccharide against Newcastle disease virus. J. Gen. Virol. 94, 2164–2174.
Wakamatsu, N., King, D.J., Kapczynski, D.R., Seal, B.S., Brown, C.C., 2006. Experimental pathogenesis for chickens, turkeys, and pigeons of exotic Newcastle disease virus from an outbreak in California during 2002–2003. Vet. Pathol. 43, 925–933.
Wang, J., Hu, Y., Wang, D., Zhang, F., Zhao, X., Abula, S., Fan, Y., Guo, L., 2010. *Lycium barbarum* polysaccharide inhibits the infectivity of Newcastle disease virus to chicken embryo fibroblast. Int. J. Biol. Macromol. 2, 212–216.
Wijesekara, I., Pangestuti, R., Kim, S.K., 2011. Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae. Carbohydr. Polym. 84, 14–21.
Witvrouw, M., De Clercq, E., 1997. Sulfated polysaccharides extracted from sea algae as potential antiviral drugs. Gen. Pharmacol. 29, 497–511.
Zhang, S., Wang, X., Zhao, C., Liu, D., Hu, Y., Zhao, J., Zhang, G., 2011. Phylogenetic and pathotypical analysis of two virulent Newcastle disease viruses isolated from domestic ducks in China. PLoS One 9, e25000.
Zhao, X.N., Hu, Y.L., Wang, D.Y., Guo, L.W., Yang, S.J., Fan, Y.P., Zhao, B.K., Wang, Y.L., Abula, S., 2010. Optimization of sulfated modification conditions of tremella polysaccharide and effects of modifiers on cellular infectivity of NDV. Int. J. Biol. Macromol. 49, 44–49.